SPECIFICATION

SECRETORY OR MEMBRANE-BINDING CHIMERIC PROTEIN

TECHNICAL FIELD

The present invention relates to a secretory or membrane-binding chimeric protein, a gene encoding the protein, a method for measuring or evaluating a gene transcription activity in a cell and a method for screening a drug which regulates gene expression. The invention also includes a method for screening a drug which regulates gene transcription expression and an enzyme activity of an enzyme which regulates protein modification.

The chimeric protein of the invention can take advantage of an energy transfer property between an energy-generating protein and an energy-receiving protein.

BACKGROUND ART

A phenomenon of energy transfer which occurs between a light-emitting enzyme and a fluorescent protein is a natural phenomenon which occurs in luminescent jellyfishes and luminescent mushrooms, and a mechanism thereof has been elucidated at a molecular level (Ohmiya, Y. and Hirano, T: Shining the light: the mechanism of the bioluminescence reaction of calcium-binding photoproteins. Chemistry & Biology 3:337-347, 1996). Furthermore, a *Renilla* luciferase and green fluorescent protein fusion gene which simulates this natural phenomenon has been constructed, and a method for quantitatively and qualitatively monitoring the gene expression using a luciferase activity and a fluorescent activity has been known (US Patent No. 5,976,796 and WO98/14605).

The *Renilla* luciferase is an enzyme purified from *Renilla reniformis*. This enzyme catalyzes oxidative decarboxylation of a luminescent substrate, coelenterazine in the presence of the enzyme to produce blue light with a maximum wavelength of 478 nm. However, in the natural world, the light shifts to green light having the maximum wavelength of 510 nm attributed to the energy

transfer to a green fluorescent protein present in *Renilla reniformis*. A *Renilla* luciferase gene has been already cloned, and a cDNA thereof has been shown to be useful as a reporter gene for measuring a transcription activity of a gene.

The fluorescent protein sometimes coexists with the light-emitting enzyme as those in the luminescent jellyfish and luminescent *Renilla* whereas it sometimes singly exists as that in cactuses. A green fluorescent protein purified from the luminescent jellyfish, *Aequorea victoria* receives blue light from a photoprotein, and converts this to green light. This gene has been cloned, and is a strong reporter gene in various biological systems (bacterial, fungal and mammalian tissues) because green fluorescence is emitted by blue excitation light without need of a cofactor when a cDNA thereof is expressed in a cell. In modified types of a wild-type green fluorescent protein, there are a modified one having a shift toward a red with blight luminescence and a modified one whose stability is improved in mammalian cells. A cDNA of a red fluorescent protein has been also cloned from a naturally occurring coral, and is also useful as the reporter gene.

A construct of the *Renilla* luciferase and green fluorescent protein fusion gene previously invented is distributed in an entire cytoplasm depending on a character of *Renilla* luciferase, and is not localized. A luminescent substrate of the *Renilla* luciferase has no cell permeability, and the gene expression can not be detected unless the cells are once lysed.

A luminescent crustacean, marine ostracod, *Vargula hilgendorfii* and its related species *Cypridina noctiluca* have a secretory light-emitting enzyme, and a cDNA of the *Vargula* luciferase has been already cloned. It reacts with a marine ostracod luminescent substrate, Cypridina luciferin to emit blue light with a maximum luminescent wavelength of 460 nm. A gene transcription activity can be measured without lysing the cells because the cloned cDNA works as the reporter gene and the light-emitting enzyme is secreted out of the cells. The secretion of a

protein from the cells can be visualized by image-analyzing of this secretory light-emitting enzyme. Meanwhile, it emits the blue light, but it has been never practically applied as a donor protein of energy transfer.

It is an object of the present invention to construct a secretory or membrane-binding protein fusion having an energy transfer property, make and use a construct having two functions of an energy-generating protein and an energy-receiving protein measurable out of a cell or on a cell surface. A gene transcription activity can be measured out of the cell as the energy transfer property, and together, a secretory pathway from an inside to an outside of the cell can be evaluated by the energy transfer property. A peptide three dimensional structure information can be obtained by inserting a monitor peptide between the energy-generating protein and the energy-receiving protein or inside the energy-generating protein or the energy-receiving protein of the secretory or membrane-binding chimeric protein, and using a change of the energy transfer by cutting the monitor peptide as an indicator. In particular, when a light-emitting enzyme and a fluorescent protein are used as the energy-generating protein and the energy-receiving protein, a fusion construct capable of measuring fluorescence without using excitation light can be made because the fluorescent protein is excited with the light emitted from the light-emitting enzyme and a color of the emitted light is changed by the energy transfer.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1a shows a construction of a luminescent fluorescent fusion protein vector in which a *Vargula* luciferase gene (Vluc) and a mutant yellow fluorescent protein gene (EYFP) amplified by PCR were inserted into an expression vector for mammalian cells, pEF-BOS.

Fig. 1b shows a luminescent fluorescent fusion protein of a *Vargula* luciferase gene (Vluc) and a mutant yellow fluorescent protein gene (EYFP) inserted into an expression vector for

mammalian cells, pEF-BOS.

Fig. 2 shows Western blot analyses in and out of cells in which a secretory luminescent fluorescent fusion protein (Vluc-EYFP) is introduced.

Fig. 3 shows fluorescent images of Cos cells in which a luminescent fluorescent fusion protein molecular probe of a secretory (Vluc-EYFP) type or a non-secretary (Rluc-EYFP) type is introduced.

Fig. 4 shows a comparison of luminescence activities of a *Vargula* luciferase alone (Vluc) and a luminescent fluorescent fusion protein (Vluc-EYFP).

Fig. 5 shows luminescence spectra of a light-emitting enzyme alone and a light-emitting enzyme/fluorescent protein fusion. A peak obtained by energy transfer was conformed to a fluorescent spectrum of a fluorescent protein. 1) Luminescence of Vluc alone, 2) luminescence of Vluc-EYFP, and 3) luminescence of EYFP.

Fig. 6 shows changes of energy transfer efficiency (changes of luminescence spectra) when a different monitor peptide was inserted in a light-emitting enzyme/fluorescent protein fusion.

Fig. 7 shows predicted secondary structures and hydrophobicity of inserted peptides 1 and 2.

Fig. 8 shows a scheme of the present invention. There exist a secretory type and a membrane-binging type in energy-generating proteins. In the chimeric protein of the invention, energy transfers from the energy-generating protein to an energy-receiving protein (black arrow), and it becomes possible to detect the energy such as light (white arrow) from the energy-receiving protein. Meanwhile, when a three dimensional structure of the chimeric protein is changed by binding an external factor to a monitor peptide or cleaving the monitor peptide, the energy from the energy-generating protein does not reach the energy-receiving protein.

Fig. 9 shows a system example of a secretory chimeric protein. In Fig. 9, the secretory protein is an energy-generating

protein.

Fig. 10 shows a system example of a membrane-binding chimeric protein. In Fig. 10, a membrane-binding region is present inside an energy-generating protein. The membrane-binding region may be in a monitor peptide or an energy-receiving protein.

DISCLOSURE OF THE INVENTION

As a result of an intensive study for solving the above problems, the present inventor has constructed a gene where an energy-generating protein and an energy-receiving protein are fused, and thereby completed the present invention.

The present gene was obtained by fusing the proteins already described in a gene database, but this combination is unknown. As described later, the present inventor has first expressed/analyzed a fusion construct and demonstrated that it comprises three properties, i.e., a secretory or membrane-binding property, an energy-generating (e.g., luminescence) property and an energy-receiving (e.g., fluorescence) property. The present inventor has also first demonstrated that energy transfer arises even in a combination of a secretory light-emitting enzyme (*Vargula* luciferase) which is not originally present in the biological world with a fluorescent protein (YFP).

(1) A secretory or membrane-binding chimeric protein composed of an energy-generating protein and an energy-receiving protein linked one another wherein energy transfer can arise between the energy-generating protein and the energy-receiving protein.

(2) The secretory or membrane-binding chimeric protein according to (1) having any structure of the following 1) to 6):

1) [secretory energy-generating protein]-[energy-receiving protein];

2) [secretory energy-receiving protein]-[energy-generating protein];

3) [membrane-binding energy-generating protein]-[energy-receiving protein];

4) [membrane-binding energy-receiving protein]-[energy-generating protein];

5) [signal peptide]-[energy-generating protein]-[energy-receiving protein]; and

6) [signal peptide]-[energy-receiving protein]-[energy-generating protein].

(3) The chimeric protein according to (1) wherein a monitor peptide is introduced between the energy-generating protein and the energy-receiving protein or inside the energy-generating protein or inside energy-receiving protein so as to retain an energy-generating property or an energy-receiving property, and the energy transfer is inhibited by cleaving the monitor peptide.

(4) The chimeric protein according to any of (1) to (3) wherein the energy-generating protein is a photoprotein.

(5) The chimeric protein according to (4) wherein the photoprotein is luciferase.

(6) The chimeric protein according to any of (1) to (3) wherein the energy-receiving protein is a fluorescent protein or a colored protein.

(7) The chimeric protein according to (6) wherein the fluorescent protein is GFP, YFP, BFP, CFP, DsRED or RFP.

(8) The chimeric protein according to (1) having an amino acid sequence represented by SEQ ID NO:1.

(9) A polynucleotide encoding the secretory or membrane-binding chimeric protein according to any of (1) to (8), or a complementary chain thereof.

(10) A vector comprising the polynucleotide according to (9).

(11) A transformant transformed with the vector according to (10).

(12) A method for producing a secretory or membrane-binding chimeric protein including a step of culturing the transformant according to (11) in a medium, and a step of collecting the secretory or membrane-binding chimeric protein from the medium.

(13) A method for measuring (evaluating) a gene

transcription activity in a host cell, characterized in that the transformant according to (11) is cultured and energy transfer in a secretory or membrane-binding chimeric protein secreted in a medium or bound to a cell membrane is quantified.

(14) A method for screening a drug which regulates gene expression in a cell, including a step of culturing the transformant according to (11) in the presence of a drug candidate compound in a medium, and a step of quantitatively comparing energy transfer in a secretory or membrane-binding chimeric protein secreted in the medium or bound to a cell membrane in the presence or absence of the candidate compound.

(15) The method according to (14) wherein the drug which regulates the gene expression in the cell is a drug which regulates gene transcription expression or an enzyme activity of an enzyme which regulates protein modification.

(16) The method according to (14) wherein the transformant comprises a polynucleotide sequence represented by SEQ ID NO:1.

The present invention will be illustrated below.

The chimeric protein of the present invention is characterized in that intramolecular energy can be transferred. Intramolecular energy transfer means that the energy transfer arises between energy (e.g., light energy) emitted from an energy-generating protein (e.g., biological photoprotein) and an energy-receiving protein (fluorescent protein, colored protein). As a result of the energy transfer, for example, a fluorescent protein can emit fluorescence without requiring external light, and this enables to quantify a level of the energy transfer.

As the energy-generation protein, preferably a biological photoprotein (light-emitting enzyme such as luciferase) is exemplified.

As the energy-receiving protein, a fluorescent protein and a colored protein are exemplified. The energy-receiving protein is a protein capable of confirming energy reception, and for example in the fluorescent protein, the level of the energy

transfer can be quantified by measuring the fluorescence.

The present invention will be more specifically illustrated below with reference to an example using a secretory biological photoprotein as the energy-generating protein and a fluorescent protein as the energy-receiving protein.

The chimeric protein is secretory, and an existence thereof can be easily detected with fluorescence by giving luciferin out of cells. Even if the chimeric protein is a membrane-binding protein, it can be similarly detected by giving the luciferin.

In the fluorescent protein such as GFP alone, a qualitative analysis by the fluorescence is possible, but a quantitative analysis is impossible because the fluorescence is emitted by external excitation light. On the other hand, in the chimeric protein of the present invention, it is possible to quantify each modification (cleavage of monitor peptide, modification by sugar chain, etc.) based on a shift of a fluorescence wavelength thereof because the fluorescent protein emits the fluorescence by an excitation light from the biological photoprotein.

In the present invention, a monitor protein is not particularly limited as long as it does not prevent the energy transfer from the biological photoprotein to the fluorescent protein. A number of amino acid residues of the monitor peptide is usually 5 to 100, preferably 6 to 50, more preferably 6 to 20, and particularly 6 to 15. It is preferable to introduce the monitor peptide at a position at which no energy transfer arises by cleavage thereof. As an introduced position of the monitor peptide, it is exemplified that the monitor peptide is introduced between the energy-generating protein and the energy-receiving protein, or inside the energy-generating protein, or inside the energy-receiving protein. It is preferable to introduce it between the energy-generating protein and the energy-receiving protein. When the monitor peptide is introduced inside the energy-generating protein, an energy-generating property remains after the introduction and a luminescence property thereof is lost by cleaving the monitor peptide. Likewise, when the monitor

peptide is introduced inside the energy-receiving protein, an energy-receiving property remains after the introduction and an energy-receiving property (e.g., fluorescence property) thereof is lost by cleaving the monitor peptide.

If a restriction enzyme site is introduced in the monitor peptide, the production of the chimeric protein becomes easier. If a proteolytic site for a certain protease is introduced in the monitor peptide, an action of the protease in a secretory process can be quantified, and it becomes possible to quantitatively screen substances which affect functions of processing enzymes of various secretory proteins. One of the preferable monitor peptide sequences is a sequence (spacer peptide sequence 1) composed of 40 amino acid residues represented by SEQ ID NO:4.

As another monitor peptide, a sequence represented by SEQKQLQKRFGGFTGG (SEQ ID NO:10) which is a partial sequence of a naturally occurring protein where a C terminus side of R can be cleaved by a processing enzyme PC1 is exemplified. The inventor has confirmed that the chimeric protein having this monitor peptide sequence has the energy transfer property and the energy transfer property is lost by cleaving the C terminus side of R of the sequence.

On the other hand, the chimeric protein having a spacer sequence 2 which is a non-naturally occurring polypeptide produces no energy transfer.

This way, in order to enable the energy transfer between the energy-generating protein and the energy-receiving protein, it is preferable that the monitor peptide has a partial amino acid sequence of a naturally occurring polypeptide or an analogous sequence to an amino acid sequence of the naturally occurring polypeptide. By making the naturally occurring polypeptide or a partial sequence thereof the monitor peptide, obviously the property of the naturally occurring sequence (e.g., a cleaving property of various proteases including the processing enzyme [PC1, PC2, furin, proteasomes, cathepsin, thrombin, etc.], a substrate of an enzyme, an agonist/antagonist of receptor, or a

binding property of a binding factor such as protein, sugar and low molecular substance) can be measured or evaluated based on the change of the energy transfer.

As used herein, as biological photoproteins, luciferases derived from various luminescent organisms such as *Cypridina hilgendorfii* (marine ostracod), *Acanthephya purpurea*, luminescent insects (firefly, headlight beetle, etc.), luminescent earthworm, *Latia neritoides*, *Renilla*, *Aequorea coerulescens* (aequorin) and the like are exemplified. For example, the *Vargula* luciferase is a secretory type, and thus the intact luciferase can be used as the secretory biological photoprotein. In the case of non-secretory luciferase such as luciferase from *Renilla*, it can be also used as the secretory biological photoprotein by introducing a signal peptide into an N terminus side.

As the fluorescent protein, a green fluorescent protein (GFP), a yellow fluorescent protein (YFP), a blue fluorescent protein (BFP), a cyan fluorescent protein (CFP), DsRED, a red fluorescent protein (RFP) and the like are exemplified.

As the colored protein, phycocyanin and phycoerythrin are included.

The fluorescent protein or colored protein of the present invention is selected such that the light emitted from the biological photoprotein has an excitation wavelength of the fluorescent protein or an absorption wavelength of the colored protein. Such combinations include the followings.

Table 1

Biological photoprotein	Fluorescent protein or colored protein
Vargula luciferase	GFP, YFP, BFP, CFP, DsRED, RFP
Firefly luciferase	DsRED, phycocyanin, phycoerythrin
Luminescent dinoflagellate luciferase	GFP, YFP, BFP, CFP, DsRED, RFP
Headlight beetle luciferase	DsRED
Renilla luciferase	GFP, YFP, BFP, CFP, DsRED, RFP
Aequorin	GFP, YFP, BFP, CFP, DsRED, RFP

The secretory biological photoprotein and the fluorescent protein may be directly linked, or may be linked through the

monitor peptide between the both.

The chimeric protein of the present invention includes the proteins represented by the following 1) to 3).

1) A secretory chimeric protein where a fluorescent protein is attached to the C terminus of a secretory biological photoprotein. One preferable embodiment of the protein is the protein represented by the amino acid sequence in SEQ ID NO:1.

2) A fused construct represented by an amino acid sequence having one or more deletions, substitutions, or additions in the amino acid sequence in SEQ ID NO:1, and having a secretory property, a light-emitting enzyme activity, a photoprotein activity and an energy transfer activity.

3) A protein where a mutation is introduced into the protein of the above 1) at a level at which the secretory property, the light-emitting enzyme activity, the photoprotein activity and the energy transfer activity are not lost. Such a mutation includes an artificial mutation in addition to a naturally occurring mutation (e.g., allele). A procedure for causing the artificial mutation can include a site-directed mutagenesis method (Nucleic Acids Res., 10:6487-6500, 1982), but is not limited thereto. A number of mutated amino acid residues is not limited as long as the secretory, light-emitting, fluorescent activities and the energy transfer property are not lost, but is preferably within 20, more preferably within 15, still more preferably within 10 and most preferably within 5 amino acid residues in the light-emitting enzyme and fluorescent protein portions. In the monitor peptide to which the light-emitting enzyme and the fluorescent protein are attached, the substitution, the deletion, the addition or the insertion can be optionally introduced for 1 to 100 amino acid residues. When the mutation is introduced by such a substitution, deletion, addition or insertion, it can be determined whether the protein in which the mutation has been introduced retains the light-emitting/fluorescent activities, by examining the light-emitting/fluorescent activities of the protein.

The polynucleotide of the present invention includes a polynucleotide encoding the secretory or membrane-binding chimeric protein where the fluorescent protein has been attached to the C terminus or N terminus side of the secretory biological photoprotein. One preferable embodiment of the polynucleotide includes a DNA represented by a nucleotide sequence described in SEQ ID NO:1 or 2, or a DNA which hybridizes with a complementary chain thereof under a stringent condition. A protein encoded by the DNA is a fused molecule having the secretory activity, the light-emitting enzyme activity, the fluorescent protein activity and the energy transfer activity.

The protein encoded by the above DNA is a protein having a secretory light-emitting enzyme activity, the fluorescent protein activity and the energy transfer activity, obtained by taking advantage of hybridization of DNA one another. Herein, the "stringent condition" refers to a condition where a specific hybridization is formed whereas non-specific hybridization is not formed. Such a condition is about "1 x SSC, 0.1% SDS and 37°C", preferably about "0.5 x SSC, 0.1% SDS and 42°C" and more preferably about "0.2 x SSC, 0.1% SDS and 65°C". A DNA obtained by hybridization with the polynucleotide described in SEQ ID NO:1 or 2 usually has high homology with a DNA represented by the nucleotide sequence described in SEQ ID NO:1 or 2. The high homology indicates 60% or more, preferably 75% or more, more preferably 90% or more and particularly 95% or more homology.

The protein of the present invention can be obtained by incorporating a gene of the invention described later into an expression vector and expressing in appropriate host cells. As the expression vector, for example, pBT-VL-mp-YFP (VL, mp and YFP represent the *Vargula* luciferase, the monitor peptide and the yellow fluorescent protein, respectively) can be used. The host cells include, eukaryotic cells such as mammalian cells and yeast, and prokaryotic cells such as cells of *Escherichia coli*, *Bacillus subtilis*, algae and fungi, and any of them may be used. As the preferable host cell, a mammalian cultured cell, COS7 cell line

(in this system, it is important to experience a protein synthesis process and a protein modification process of a mammalian system, and these processes are monitored) and the like can be used.

The gene (polynucleotide) encoding the preferable chimeric protein of the present invention is:

1) a gene having a nucleotide sequence described in SEQ ID NO:1; and

2) a gene having a DNA which hybridizes with a DNA represented by the nucleotide sequence described in SEQ ID NO:1 or a DNA complementary thereto under the stringent condition.

Schematic views of the system of the present invention are shown in Figs. 8 to 10. As shown in Fig. 8, in the chimeric protein of the invention, in the case of no structural change of the monitor peptide, the energy transfers from the energy-generating protein to the energy-receiving protein as shown by a black arrow, and it becomes possible to detect the energy (white arrow) such as light from the energy-receiving protein. On the other hand, when the three dimensional structure of the chimeric protein is changed by binding an external factor to the monitor peptide or cleaving the monitor peptide, the energy from the energy-generating protein does not reach the energy-receiving protein. The energy emitted from the chimeric protein is different in energy (white arrow in Fig. 8) through the energy-receiving protein and energy (black arrow in Fig. 8) directly emitted from the energy-generating protein. Therefore, it is possible to quantify a level of an effect of a factor which affects the chimeric protein by measuring the energy from the chimeric protein. For example, when the factor is a candidate compound for a drug, the chimeric protein of the invention is useful for a screening system of the drug as shown below.

As shown in Fig. 9, when the energy-generating protein in the chimeric protein of the present invention is a secretory protein, it is useful for monitoring a change on a secretory pathway of a processing enzyme activity. As is also shown in Fig.

10, when the chimeric protein of the invention is a membrane-binding protein, it is useful for monitoring an intracellular or extracellular change around the membrane.

(Screening method)

By culturing a transformant in which the gene encoding the chimeric protein of the present invention is incorporated, it is possible to quantitatively measure (evaluate) a gene transcription activity in a host cell, and screen a drug which regulates the gene expression in the cell.

By introducing the chimeric protein of the present invention into the cells and tracking a fluorescence intensity and a shift of a wavelength, it can be examined whether mechanisms of synthetic rate of a protein from mRNA, folding and processing of the synthesized protein, membrane-binding or secretion of the protein in the cells are normally operated.

Therefore, by comparing results obtained by culturing the transformant capable of expressing the chimeric protein of the invention in the presence or absence of a candidate compound for a drug, it can be found what action the candidate compound gives to a protein expression system, and it is possible to select the candidate compound which acts upon the protein expression system(gene expression system).

Compared subjects include a total amount of fusion protein expression, comparison of an intracellular and extracellular expression amounts of the fusion protein, shift levels of the fluorescence wavelength of the fusion protein for the fluorescent protein, levels of a cleavage or a sugar chain bond of the monitor peptide, and the like.

For example, some patients with diabetes have a processing abnormality from proinsulin to insulin, i.e., can not synthesize active insulin due to a mutation of an amino acid residue at a cut site and a low activity of a limited proteolytic enzyme. A drug which normalizes such a processing abnormality is useful as a drug for treating the diabetes.

As peptide hormones whose common precursor is pro-

opiomelanocortin (POMC), there are ACTH, β-lipotropin (βLPH), α and β melanocyte stimulating hormones (MSH), enkephalin and endorphin. By affecting a cleaving process from POMC to an active peptide, it is possible to use as a screening system of an anti-inflammatory agent with a cut out of ACTH as an indicator or an analgesic agent with a cut out of endorphin as an indicator.

Since the secretory chimeric protein of the present invention is secreted out of the cells, the biological photoprotein and the fluorescent protein can be each independently quantified by adding a luminescent substrate (luciferin) in the culture medium. Levels of luminescence of the biological photoprotein and the fluorescent protein are remarkably affected by cleavage of the fluorescent protein, cleavage of the monitor protein and sugar chain modification, and thus, it can be quantitatively evaluated how the drug candidate compound affects the gene expression system by the screening system of the present invention.

Since the chimeric protein of the present invention is secreted out of the cells, a luminescence spectrum and a luminescence activity can be measured by adding the luminescent substrate (luciferin) into the culture medium without lysing the cells, and the energy transfer which arises between the biological photoprotein and the fluorescent protein can be quantified. The secreted fusion protein is highly modified through a physiological secretory process in the cells. The energy transfer which arises between the biological photoprotein and the fluorescent protein is remarkably changed and a shape of the luminescence spectrum is changed by cleavage and sugar chain modification of the monitor peptide. For example, the energy transfer disappears along with the cleavage of the monitor peptide, and the energy transfer is increased/decreased by the sugar chain modification. Thus, by the screening system of the present invention, it can be quantitatively evaluated what levels of the effect the drug candidate compound gives to an enzyme activity of a protein modifying enzyme which cleaves or modifies

a sugar chain and the gene expression thereof.

BEST MODE FOR CARRYING OUT THE INVENTION

The present invention will be illustrated in more detail with reference to the following Examples, but the invention is not limited thereto.

Example 1.

Fragments of *Vargula* luciferase (hereinafter, sometimes abbreviated as "VL" or "Vluc") gene (Thompson, E. M., Nagata, S. & Tsuji, F. I. Cloning and expression of cDNA for the luciferase from the marine ostracod *Vargula hilgendorfii*. Proc. Natl. Acad. Sci. USA 86:6567-71 ,1989) and a mutant yellow fluorescent protein (EYFP) gene derived from a luminescent jellyfish were amplified by a polymerase chain reaction (PCR method), and inserted into an expression vector for mammals to construct a luminescent/fluorescent fusion protein gene. Fig. 1 shows a map of the vector and an alignment of gene portions. When the Vluc fragment was amplified by PCR, using a primer 1 (5'-(*Hind*III-*Bst*XI) : CACAAGCTTCCATTGTGCTGGATGAAGATAATAATTCTGTCTGTTATATTGGC-3'; primer 2 (5'-(*Bam*HI): TGTGGATCCTTGACATTCAGGTGGTACTTCTAG-3', an initiation codon was given to the N-terminus of Vluc, a termination codon was deleted from the C-terminus, and a linker sequence containing a *Bam*HI site was introduced. Meanwhile, for the PCR amplification of the EYFP fragment, a primer 3 (5'-(*Hind*III-*Not*I-*Bam*HI): CAAGCTTGCGGCCGCAGGATCCGTGAGCAAGGGCGAGGAGCTGTTCAC-3'), a primer 4 (5'-(*Bst*XI) TACCATTGTGCTGGATGGTGAGCAAGGGCGAGGAGCTG-3') were used, the initiation codon was deleted from the N-terminus of EYFP, the linker sequence containing a *Bam*HI site was ligated to the N-terminus, and the termination codon was introduced at the C-terminus. The above PCR products were sequentially inserted into a *Bst*XI site of pEF-BOS (S Mizushima & S Nagata pEF-BOS, a powerful mammalian expression vector. Nucleic Acids Research, Vol.18, No.17 P.5322) already known publicly as the expression vector for the mammals to make an expression vector, and

designated as pEF-BOS Vluc-EYFP. In this vector, the *Vargula* luciferase gene was arranged upstream, the mutant yellow fluorescent protein gene from the luminescent jellyfish was arranged downstream, and a peptide sequence can be inserted at a *Bam*HI restriction enzyme site between them.

Example 2.

The luminescent/fluorescent fusion protein gene, pEF-BOS Vluc-EYFP was introduced into COS7 cells, and it has confirmed by Western blotting method using a light-emitting enzyme Vluc antibody and a fluorescent protein EYFP antibody that the luminescent/fluorescent fusion protein, Vluc-EYFP had been produced. Fig. 2 shows the intracellular results and the results from the extracellular medium when Vluc, EYFP and Vluc-EYFP were introduced, respectively. The Vluc (molecular weight: 63 kDa), the EYFP (27 kDa), and the Vluc-EYFP (95 kDa) were secreted out of the cells. In the intracellular expression of Vluc-EYFP, smaller size proteins were also produced whereas the secreted one appears to have a full length. Since the fusion protein is secreted, fluorescent images of the COS7 cells were observed. As a comparison, cells in which Rluc-EYFP which was a fusion of *Renilla* luciferase, Rluc with no secretory signal and EYFP had been introduced were observed. According to the fluorescent images in Fig. 3, it has been confirmed that the fluorescence of RLUC-EYFP is distributed evenly throughout the cells whereas the fluorescence of Vluc-EYFP is localized in the cells, indicating that the Vluc-EYFP protein is secreted by responding to the secretory signal of the Vluc. A luminescence activity of the secreted Vluc-EYFP was measured, and consequently the Vluc-EYFP retained about 80% luminescence activity relative to the Vluc alone as shown in Fig. 4. From the above results, it has been confirmed that the Vluc-EYFP is the fusion protein having a luminescence activity ability and a secretory ability which the Vluc has and a fluorescent ability of the fluorescent protein. The fluorescence activity, the secretory ability and the fluorescence also indicate a strength of a promoter activity, a

visualization ability of a secretory process/pathway, and a localization of the fusion protein, respectively.

Example 3.

Luminescence spectra of the Vluc-EYFP were measured. Two peaks of the luminescence spectra were observed as shown in Fig. 5 (Fig. 5-2). One was conformed to a peak of the light-emitting enzyme alone (Fig. 5-1), i.e., a maximum luminescence wavelength of 460 nm. A peak at a longer wavelength side was conformed to a peak of a fluorescent spectrum of the fluorescent protein alone (Fig. 5-3), and was also conformed to a fluorescent spectrum of the light-emitting/fluorescent fusion protein. This peak at the longer wavelength side is attributed to the energy transfer between the photoprotein and the fluorescent protein, i.e., the light emitted from the photoprotein becomes excitation light, and the light with longer wavelength, fluorescence was emitted from the fluorescent protein by the excitation light. It has been confirmed that this energy transfer does not arise even when the Vluc and the EYFP are simply mixed. Therefore, the Vluc-EYFP is a construct having the secretory property, where the light-emitting/fluorescence property and the energy transfer property between the photoprotein and the fluorescent protein are retained.

Example 4.

Two peptide sequences were inserted, respectively at *Bam*HI restriction enzyme site of a linker sequence moiety of the luminescent/fluorescent fusion protein gene. Fig. 6 shows luminescence spectra for the inserted peptide 1 and the inserted peptide 2. In the protein with the inserted peptide 1, the energy transfer arose and a small peak at the longer wavelength side was observed. However, in the protein with the inserted peptide 2, no energy transfer arose. From this result, it has been demonstrated that the energy transfer depends on the inserted peptide sequence. A secondary structure of each peptide was predicted, hydrophobicity was analyzed, and consequently it was anticipated that a three dimensional structure of each peptide was different one another (Fig. 7). It has been demonstrated that three

dimensional structure information of the inserted peptide can be obtained by the use of this energy transfer difference as an indicator.

The present invention provides the monitor protein, the gene encoding it, and the gene which controls the expression of the present enzyme. This fusion protein has the secretory property and biological luminescence activity of the *Vargula* luciferase and simultaneously has the fluorescence activity of the fluorescent protein. The *Vargula* luciferase-fluorescent protein fusion protein can be utilized as multiple markers for quantitatively monitoring an expressed amount of the gene and the secretion out of the cells by the enzymatic activity and a position information of secretory sites in the cells by the fluorescence. Furthermore, it can be utilized as a sensor for obtaining a functional change information of the three dimensional structure, the cleavage and the sugar addition of the inserted peptide because the energy transfer efficiency is changed by arranging the peptide between the luciferase and the fluorescent protein.